Role of Steroid Therapy after Ischemic Stroke by N-Methyl-D-Aspartate Receptor Gene Regulation

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Background: Stroke is the main cause of cerebrovascular disease mortality. Prolonged stimulation of N-methyl-D-aspartate (NMDA) receptor subtypes by the accumulation of glutamate neurotransmitter in the extracellular space after a stroke could activate cell death pathways. It is reported that progesterone provides different mechanisms of neuroprotection and could be considered as a candidate for stroke treatment. This study aimed to investigate progesterone impact on the expression of NMDA receptor subunits NR1, NR2 (A and B), NR3 (A and B) after an experimental model of ischemic stroke which is followed by an in silico analysis. *Methods:* Progesterone was introduced subcutaneously after transient middle cerebral artery occlusion in male rats. After a period of reperfusion, a set of behavioral tests was performed to evaluate the postischemic neurological deficits. The 2,3,5-triphenyltetrazolium chloride staining method was done for quantification of infarct volume and gene expression analysis was performed in the penumbra region using reverse transcription polymerase chain reaction for NMDA receptor subunits. An AutoDock tool was employed to perform molecular docking analyses for evaluation of progesterone interaction with NMDA receptor. *Results:* Cerebral ischemia caused a significant downregulation in NR1, NR2A, NR2B and a profound upregulation of NR3B in cortical penumbra region. Treatment with progesterone resulted in upregulation of NR1, NR2A, and NR3B which could explain a possible the neuroprotection of steroids via binding to NMDA glutamate receptor. In addition, in silico analysis revealed that progesterone could strongly interact with NR1/NR2B and NR2A. *Conclusion:* The findings elucidate a new aspect of the neuroprotective mechanism of progesterone via NMDA receptors gene regulation.

Keywords: Stroke—NMDA receptor—Progesterone—Gene expression

Introduction

Glutamate is the main neurotransmitter in the brain and is required for normal synaptic function; it has also an important role in neuronal growth and central nervous systems development. Glutamate-mediated excitotoxicity is one of the most important mechanisms that cause neurotoxicity after stroke, so understanding its molecular mechanisms can prevent ischemia damage.[1] Brain tissue is very vulnerable to ischemia that accounts for about 80% of all strokes. Even a temporary blockade of blood flow in the brain could make a progressive damage to neurons. So it is essential to understand glutamate-mediated molecular mechanisms after stroke in order to provide more effective therapeutics and decrease the stroke socioeconomic burden.[2] N-methyl-D-aspartate (NMDA) receptor is one of the most important glutamate-gated transmembrane ion channel and functions at the postsynaptic membrane of excitatory synapses in the central nervous systems. Ischemic conditions cause accumulation of

Abbreviations: CNS, central nervous systems; CBF, cerebral blood flow; CCA, common carotid artery; DMSO, dimethyl sulfoxide; ECA, external carotid artery; ICA, internal carotid artery; MCAO, middle cerebral artery occlusion; NMDAR, N-methyl-D aspartate receptor; NR, NMDA receptor; PROG, progesterone; tMCAO, transient MCAO

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glutamate neurotransmitter in the synaptic cleft which leads to overactivation of NMDA receptor subtypes (NMDARs) and increases Ca^{2+} influx to the postsynaptic neuron leading to excitotoxicity and downstream neuronal death pathway activation.[3] Previous studies showed that NMDARs' subunit location and composition could regulate neuronal survival or death.[4] It is also reported that ischemia changes the regulation of some NMDAR subunits and pharmacological manipulation of these subunits could help to identify novel therapeutic strategies. Neurosteroids have been shown to have modulatory effects on NMDARs and could induce potentiation and/or inhibition effects.[5] Recently, it has been shown that progesterone (PROG) provides multiple mechanisms of neuroprotection and could be a suitable candidate for stroke therapy.[6]

However, the effects of PROG on NMDAR subunit expression have not yet studied completely. Regarding the importance of NMDAR subunit composition in the determination of receptor function, studying the influence of PROG on their expression could result in more elucidated knowledge about the neuroprotective mechanism of the hormone. Therefore, this study aimed to investigate the effect of PROG treatment on behavioral outcomes, infarct volume, and gene expression of NR1, NR2A, NR2B, NR3A, and NR3B subunits of NMDAR in an experimental model of ischemic stroke in rats. In addition, we performed an in silico analysis to assess the interaction of PROG with NMDAR receptor.

Materials and Methods

Experimental Groups

Male Wistar rats (16 weeks old, 240-300 g) were obtained from domestic laboratory animal house (Kashan University of Medical Sciences Animal House) and maintained in a 12 hour light/dark cycle with free access to food and water. Experimental procedures were approved by local Ethical Committee and were carried out in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes. Animals were randomly divided into three groups: (1) control vehicle, (2) vehicle-treated ischemia/reperfusion (I/R) group (ischemia), and (3) PROG-treated I/R group (ischemia + PROG). All tests were performed according to guide to use and care of Laboratory Animals and approved by the ethics committee of the Kashan University of Medical Sciences, Kashan, Iran.

Focal Ischemia Animal Model and Hormone Treatment

Transient middle cerebral artery occlusion (tMCAO) was used to prepare experimental stroke as described previously.[7] Briefly, anesthesia was induced by 5% isoflurane using a vaporizer (Eickemeiyer, Germany) for 2-3 minutes and maintained at 2%-3% during surgical procedures. Body temperature was maintained at 37 °C ± 0.5 °C using a warm pad (Narco Bio-Systems, Houston, Texas) and rectal temperature was monitored during surgery (Nahavandi). To assure a successful occlusion of the middle cerebral artery, cerebral blood flow (CBF) on both ipsi- and contralateral hemispheres was measured by Laser-Doppler Flowmetry (Moor Instruments VMS-LDF2, UK). Probes were positioned and fixed at ~4-5 mm lateral and ~1-2 mm posterior to the bregma on right and left drill-thinned skull. A small midline neck incision was made to expose the left common carotid artery (CCA), internal carotid artery, and external carotid artery. Vagus nerve was separated from the CCA and a small incision performed on CCA using micro scissors (Fine Science Tools, Canada). Then a silicon-coated monofilament (404356PK10Re, Doccol, Sharon, Massachusetts) introduced into the lumen of CCA and forwarded to internal carotid artery at the bifurcation until a significant drop in CBF (>50% compared to baseline value). After 1 h occlusion, monofilament was removed carefully and the reperfusion was occurred in the corresponding ischemic region for 24 h. Sham animals were operated to the same surgical steps, but the catheter did not introduce into the CCA.10 mg/kg PROG (Sigma, Germany) was initially diluted in pure ethanol, then diluted again in sesame oil to the final volume of 500 μL and was given as neck depots immediately after MCAO induction. Dosage of the hormone has been previously reported to gain the rapid physiologically high steroid plasma amounts ($P \sim 70$ ng/ml) with the maximum of short-term protection.[7] Sham animals received an appropriate amount of ethanol/sesame oil mixture.

Behavioral Testing

Behavioral evaluation, including motor and sensory tests, was applied to evaluate postischemic neurological deficits 24 h after tMCAO.[8] The following six parameters were evaluated:

1- Spontaneous activity: rats were placed in an open environment (60 cm × 90 cm sized cage) and behavior was monitored for 3 minutes (scores: 0 = not moving; 1 = hardly moving around, but not rising up in the cage's wall; 2 = moving, but undecided to travel all the cages, ultimately rising to at least one side of the cage; 3 = moving around, exploring the environment, and approaching at least 3 walls of the cage).
2- Forepaw outstretching: proximal part of the tail was taken to evaluate outstretching of forelimbs (scores: 0 = right forelimb entirely constricted; 1 = right side moves slightly; 2 = outstretching in the right side is less than left side; 3 = symmetrical outstretching in both forelimbs).

3- Climbing ability: animals were placed on a ladder observing how they move to the top (scores: 1 = unable to climb or hold the wire; 2 = right side is weak during climbing; 3 = climbing easily and clutching the stair tightly).
4- Spontaneous activity: walking was checked (scores: 0 = not walking; 1 = walking shift to the right; 2 = right circling; 3 = walking straight ahead).
5- Trunk proprioception: each side of the body was touched with a blunt stick and the response to the stimulus was assess (scores: 1 = not responding to the stimulus on right side; 2 = response to the stimulus on right side slowly; 3 = moving the head toward the stimulus and a symmetrical manner on both sides).
6- Head proprioception: brushing was applied to the vibrissae (scores: 1 = no response to the stimulus on the right side; 2 = slow reaction to the stimulus on the right side; 3 = turning the head to the stimulus side). Finally, all individual test scores were summed, and a total minimum score of 3 and a total maximum score of 18 was feasible.

2,3,5-Triphenyltetrazolium Chloride Staining and Quantification of Infarct Volume

Twenty-four hours after tMCAO, rats were deeply anesthetized by intraperitoneal injection of 10% chloral hydrate; then sacrificed. Brains were removed and cut into 2 mm coronal sections quickly by a rat brain matrix (Zivic Instruments, Pittsburg, Pennsylvania). Brain slices were soaked in a normal saline solution containing 1% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) and incubated at 37° for 20 minutes. In metabolic active tissue, TTC is reduced into red 1,3,5-triphenylformazan in the presence of mitochondrial dehydrogenase whereas infarct area remains white.[9] After arranging of the brain slices in a frontal to occipital orientation, photos were taken using a digital camera (Nikon, Japan). The ImageJ 1.44 software (NIH, Bethesda, Maryland, USA) was used for evaluation of the infarct area. Infarcted areas of ischemia-affected sections were measured (mm2) and multiplied by the thickness of sections (2 mm) in order to get the infarction volume of each brain.[10]

Analysis of Gene Expression in Cortical Peri-infarct Region

For gene expression analysis, samples were taken from tissues related to the peri-infarct area distinct in TTC-stained sections, and tissues from the corresponding contralateral area in the nonischemic hemisphere were used as the control as described previously.[9, 11] Total RNA isolation from the peri-infarct area was performed using the Peqgold total RNA extraction reagent (Peqlab, Germany) and complementary DNA (cDNA) synthesis was performed by cDNA synthesis kit (Invitrogen). cDNA was diluted in 1:10 ratio and polymerase chain reaction (PCR) was carried out in a 96 × 0.2 ml plate (Invitrogen, Carlsbad, California) containing a 5 μl SensiMix master mix (Bioline, Germany), 1 μl of primers, 2 μl distilled water, and 2 μl of diluted cDNA. reverse transcription PCR (RT-PCR) technology (BioRad, Germany) with a standardized protocol was used to measure the gene expression as described previously.[12] Relative standard curve method was performed by utilizing the housekeeper gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) as a reference gene. Primers used in the study are summarized in Table 1.

Statistical Analysis

A total number of 22 rats were used in this study, including sham (n = 6), ischemia (n = 8), and PROG treated (n = 8). All data are presented as means ± SEM and analyzed with SPSS software (Ver. 22.0). For comparison of cerebral blood flow (CBF) data, repeated measurement ANOVA was used. The behavioral scores were analyzed using Kruskal–Wallis test. Ratios from real-time relative quantity data were compared using one-way ANOVA and post hoc Tukey's test. Differences were considered to be statistically significant if $p < 0.05$.

In silico Analysis

Since neurosteroids could have modulating effects on NMDA receptor, in this section of the study we evaluated the interaction of PROG with NMDA receptor by a molecular docking approach. For this purpose, at first X-ray crystallographic structures of NR1 (ID: 4NF8), NR2A (ID: 2A5S), NR2B (ID: 4NF8), NR3A (ID: 2AC7), and NR3B (ID: 2RCA) molecules were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (https://www.rcsb.org/). All of these structures were related to rat species (*Rattus norvegicus*) and they were selected with resolution less than 2. The stability of structures was approved by Ramachandran plot which prepared by RAMPAGE web server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). To create the apoprotein structure, some molecules such as water, ligands, etc. were removed from protein data bank structures by Chimera UCSF Chimera (Ver.1.11.2) software and final the structures were assessed by ConTEXT software (Ver.0.98.6) to ensure proper trimming. Finally, hydrogen and charge were added to all structures for docking purpose. In addition, 3-dimensional structure of PROG (ID: 5994), glycine (ID: 750), and glutamate (ID:33032) were obtained from PubChem database. Treatment of these structures including energy

Table 1. *Primer sequences, PCR product size, and annealing temperatures (AT) in real-time PCR*

Gene	Accession no.	Primer sequence (5'→3')	TA °C	Size (bp)
NR1	NM_001287423	AGTGGAACGGAATGATGGGCGA ACTGAAGCGGTCCAGCAGGTA	57	262
NR2A	NM_012573	GAGCCAGATGACAACCACCT TCTTGAGGATGTCGATGCAG	58	200
NR2B	NM_012574	CCAAGAGGAGGAAACAGCAG TGAGGCGAGTTCTCCTTTGT	56	184
NR3A	NM_138546	TAGGAAAGCCATTTGCCATC GAAGTGCTTGATGCCCATTT	56	202
NR3B	NM_133308	ACCGTGGCACTGTCTTCTCT TCAAAGGTTTTGTCCCCAAC	60	177
HPRT	NM_012583	GCTGGTGAAAAGGACCTCT CACAGGACTAGAACACCTGC	60	284

minimization of ligand, making the correct form of the tautomer, adding hydrogen atoms to ensure the valence of heavy atoms, and adding partial charges were performed by AutoDockToolsoftware(Ver.1.5.6)fordockingprocedure.

For docking simulation, the proteins and the ligands were loaded into AutoDock Tools. Gasteiger partial charges were assigned after merging nonpolar hydrogen and torsions applied to the ligands by all rotatable bonds. Docking calculations were carried out on the protein models. Polar hydrogen atoms Kallman charges and solvation parameters were added with the aid of AutoDock Tools. AutoDock Tool offers the option of three search algorithms to explore the space of active binding with different efficacy. We used the Lamarckian genetic algorithm (LGA) in this study. Docking was performed for each of the subunits of NMDA and near the NR1NR2A and NR1NR2B dimer interface. Information on the binding of hydrogen and the grid is fully listed in Table 2. For each of them, 50 independent runs implementations are considered. Then, LigPlot+ (Ver. 1.4.5) and Chimera UCSF (Ver. 1.11.2) were used to visualize the complex protein pattern of the ligand.

Results

CBF, Behavioral Deficits, and Infarct Volume Analyses

To make sure a successful tMCAO, CBF on both ipsi- and contralateral sides was measured in both ischemic and treated groups which are described in "Materials and Methods" section. Data from Laser-Doppler flowmetry showed that after tMCAO, the regional CBF values decreased to >60% compared with pre-ischemic values. Also, this value was stable during the occlusion (Fig. 1A). As depicted in Figure 1(A), there was no significant difference between ischemic and treated groups. Mean values reduced to ~60% in both ischemic and treated groups compared with baseline values.

As shown in Fig. 1(B), tMCAO significantly reduced the maximum score from 18 in sham-operated animals to ~12 in tMCAO group. Also, scoring of each subcategory was comparable separately (data are not shown). The PROG treated ischemic rats showed significantly improved behavioral outcomes ($P < 0.01$) and reduced infarct volume ($P < 0.05$). The extensive infarct volume was observed in the striatum and cortex of control group and reduced significantly after treatment with PROG, especially in the cortex region ($P < 0.05$; Fig. 2).

Expression of NMDA Receptor Subunits in the Peri-infarct Area

The data from real-time PCR revealed that after stroke, the mRNA expression of NMDAR subunits NR1, NR2A, and NR2B were downregulated, while the expression of NR3B was upregulated significantly ($P < 0.001$). The treatment of PROG resulted in upregulation of NR1 ($P <$

Table 2. *Results of molecular docking for different subunits of NMDAR*

NMDAR subunits	LBE (kcat/mol)	Number of HB	RMSD	Residues involved in HB	CGB
NR1	−7.81	1	0.56	His12	x:19.52; y:42.464; z:38.253
NR2A	−7.97	2	0.0	Lys41, Val197	x:26.718, y:22.536, z:32.318
NR2B	−7.55	1	0.04	Val197	x:32.033; y:72.96; z:10.748
NR3A	−6.8	1	0.83	Ser144	x:10.956; y:46.687; z:0.532
NR3B	−7.24	1	0.0	His15	x:53.293; y:21.804; z:20.121
NR1NR2A	−6.07	1	0.0	Leu244	x:15.131; y:-15.16; z:35.727
NR1NR2B	−10.66	1	0.0	Glu133	x:40.472; y:75.404; z:26.083

Abbreviations: CGB, center grid box; , HB, hydrogen bonds; LBE, lowest binding energy; RMSD, Root mean square deviation.

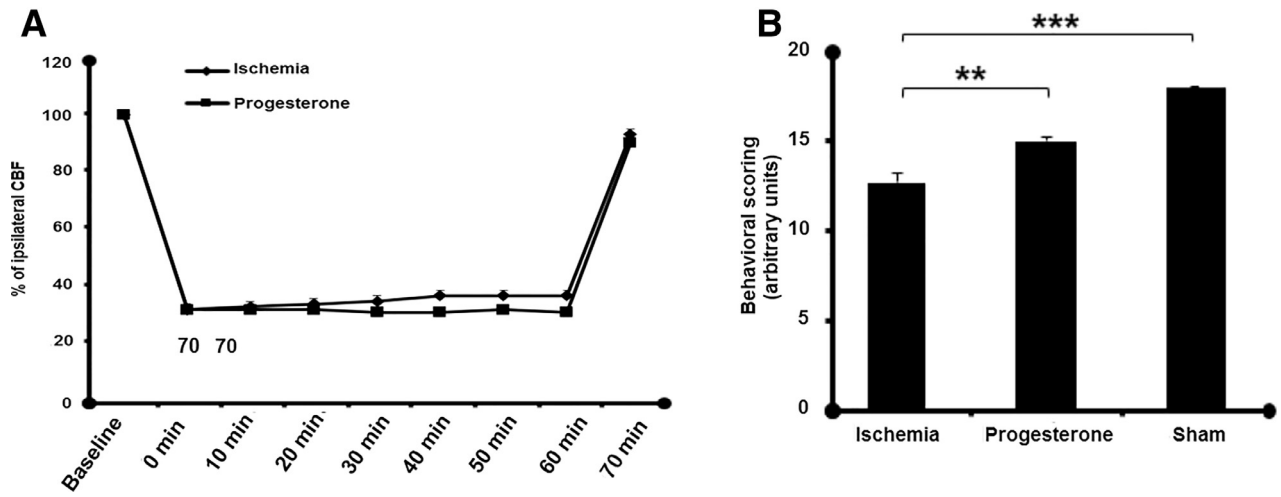

Figure 1. *Measurement of CBF over the middle cerebral artery territory before, during (60 minutes), and after tMCAO. (A) Decrease in CBF shown as percentage of baseline values and the CBF increases after tMCAO. tMCAO significantly reduced the maximum score from 18 in sham-operated animals without tMCAO to ~12. (B) The PROG-treated ischemic rats showed significantly improved behavioral outcomes (*P* < 0.01).*

0.05), NR2A ($P < 0.01$), and NR3B ($P < 0.01$) significantly. NR3A subunit expression was not influenced significantly after strokes while this subunit was downregulated after treatment with PROG (Fig. 3).

Docking Results

The complex of NMDAR including two NR1 and NR2B has been shown in Figure 4. As shown, NR1 subunit could interact with glycine and NR2B could bind to glutamate. Results of molecular docking including lowest binding energy (kcal/mol), number of hydrogen bonds, root mean square deviation (RMSD), and residues involved in H-binding are detailed in Table 2. The data revealed that the PROG could bind to NMDAR near the glycine binding site of NR2A with lowest binding energy equal to −7.97 kcal/mol (Table 2). Also, the data showed that the PROG could strongly interact with dimmer interface of NR1/NR2B heterodimer with lowest binding energy equal to −10.66 kcal/mol (Fig. 5). The 2-dimensional analysis showed that the hydrogen bond will be established between Glu113 of NR2B and oxygen linked to carbone 20 of PROG (Fig. 5).

Discussion

The vast majority of deaths from cerebrovascular diseases are due to stroke, the second leading cause of mortality and also one of the leading causes of morbidity worldwide.[13] The burden of disease is great and in order to lessen it by providing effective therapeutics, understanding the molecular mechanisms involving stroke

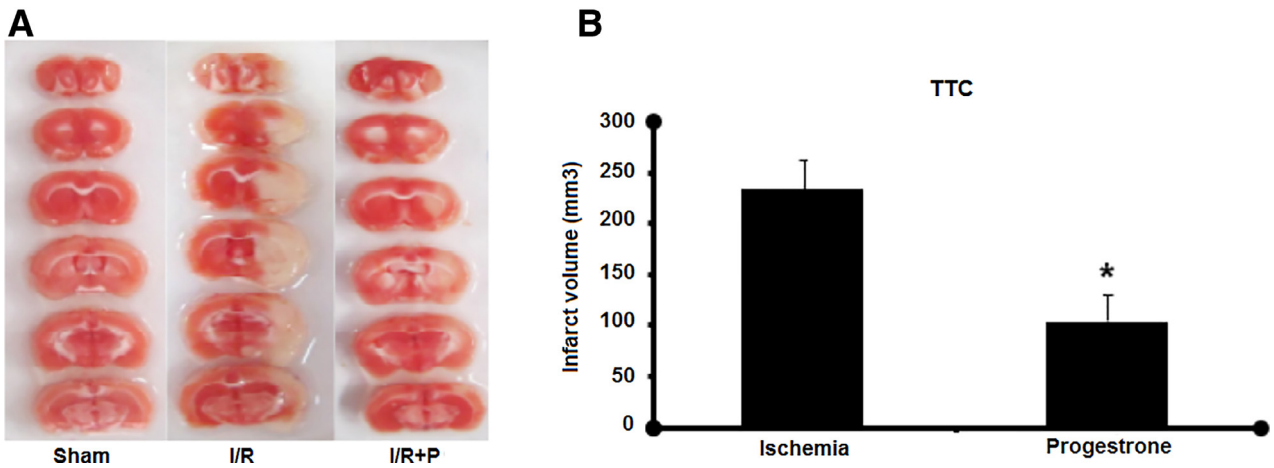

Figure 2. *Changes of infarct volume after hormone treatment. (A) The infarct volume in sham, stroke (I/R), and treated with PROG (IR+P) groups. (B) Results from the measurement of infarct volume in the ischemia and treated with PROG groups (*P < 0.05).*

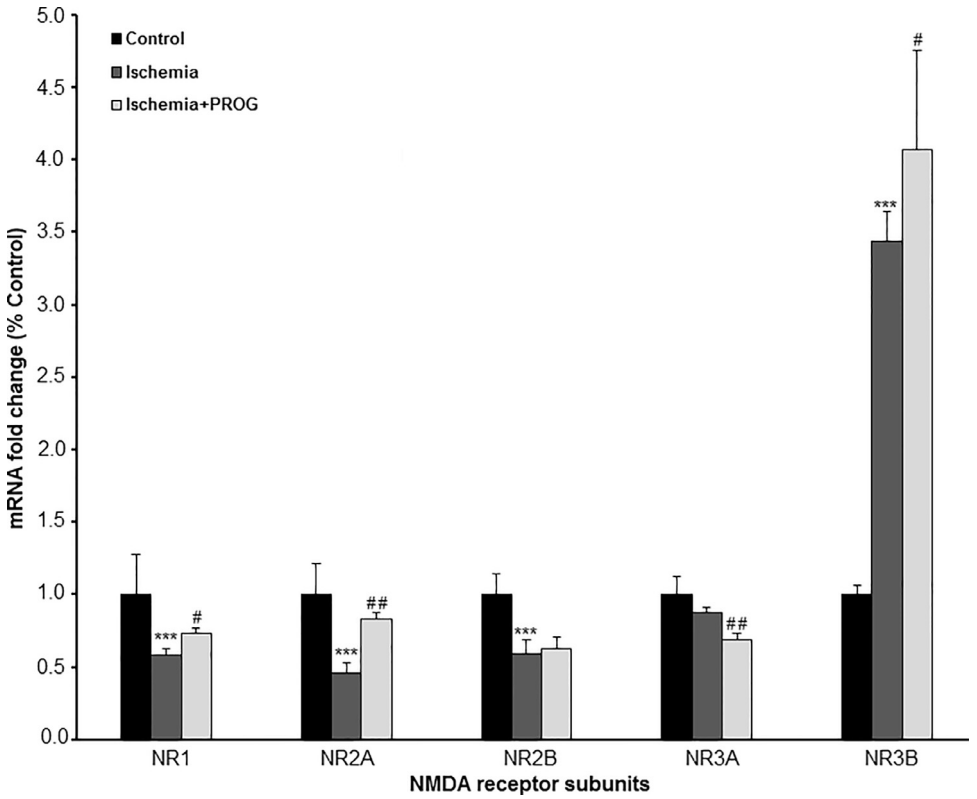

Figure 3. *Expression pattern of NMDA receptor subunits in peri-infarct area. After MCAO the gene expression of NMDAR subunits NR1, NR2A, and NR2B was downregulated, while the expression of NR3B was upregulated significantly* ($P < 0.001$). *The treatment of PROG resulted in upregulation of NR1* ($P < 0.05$), *NR2A* ($P < 0.01$), *and NR3B* ($P < 0.01$) *significantly. The NR3A expression was not influenced significantly after MCAO while this subunit downregulated after treatment with PROG. (*indicates significant P-value ischemia versus control; # indicates significant P-value ischemia + PROG versus ischemia). The P-values are indicated as # and *:* $P \leq 0.05$; *## and **:* $P \leq 0.01$; *### and ***:* $P \leq 0.001$. *(A total number of 22 rats were used in this study, including sham [n=6], ischemia [n=8], and progesterone treated [n=8]).*

seems essential. Recently, it is discussed that the PROG hormone could provide neuroprotective effects and here we aimed to investigate the possible relationship between PROG protective mechanisms after stroke with NMDA receptor. In this study, we evaluated the infarct volume after stroke and treatment with PROG. Our data revealed

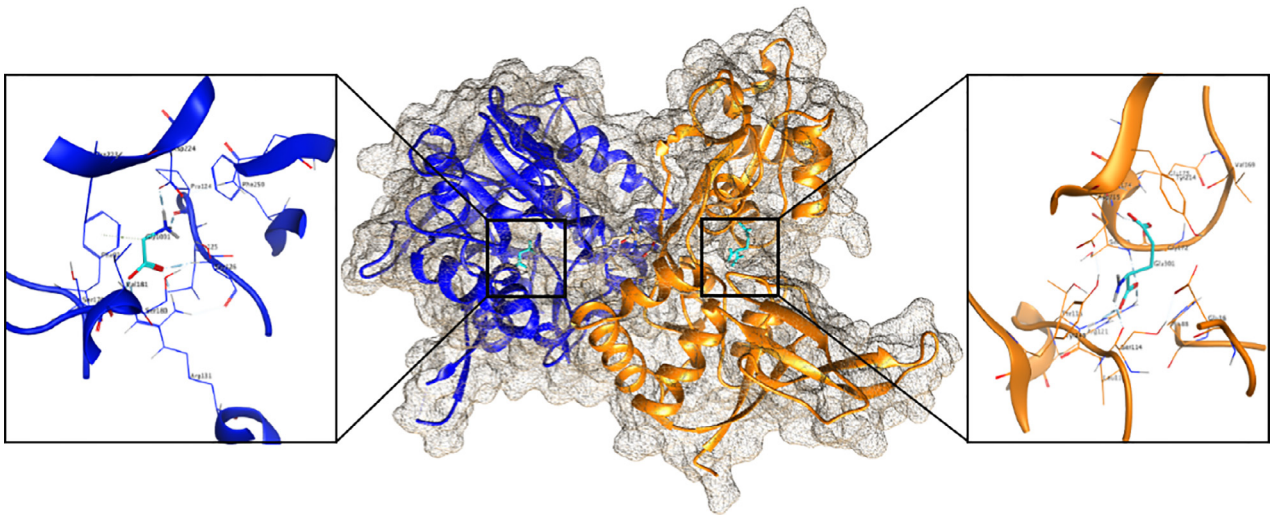

Figure 4. *Ribbon structure of NMDAR. NMDAR ligand binding domains having two subunits including NR1 (blue) binds to glycine and NR2B (orange) binds to glutamate.*

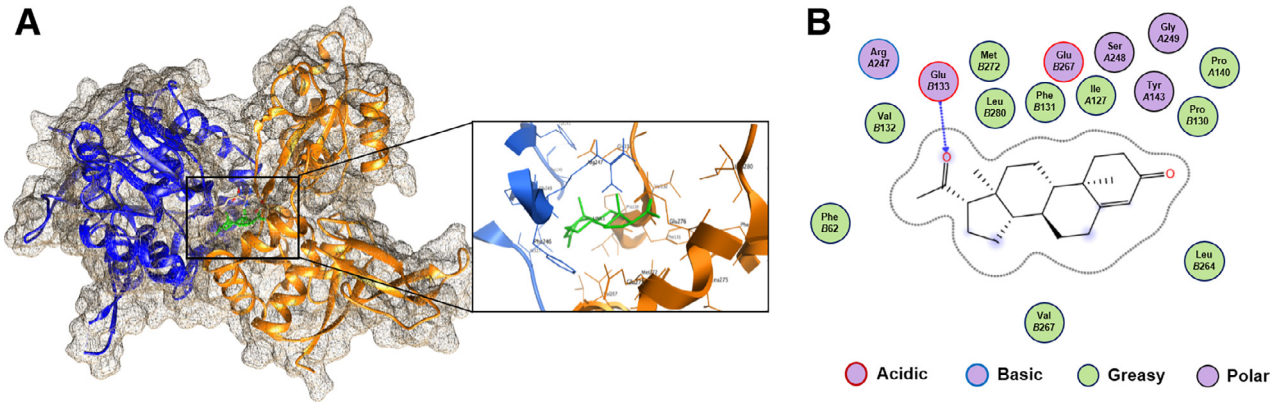

Figure 5. *Interaction of NMDAR with progesterone. (A) Rounded ribbon structure of NMDAR ligand binding domains having two subunits including NR1 and NR2B in complex with progesterone; NR1, NR2B, and progesterone indicated with blue, orange, and green, respectively. (B) The two-dimensional analysis showed an established hydrogen bound between Glu113 of NR2B and oxygen linked to carbone 20 of progesterone. The conventional hydrogen bond is shown by blue arrowhead.*

that the extent infarct volume was observed in the striatum and cortex of ischemia group. Consistent with previous reports, we showed that treatment with PROG reduces infarct volume significantly.[14, 15] Also, we analyzed stroke-induced functional damages in the rats after tMCAO. As shown in Figure 1(B), tMCAO significantly reduced the maximum score from 18 in sham-operated animals to ~12 in ischemia group. The peri-infarct area is well defined as the area adjacent to ischemic area. A broadly established definition of the peri-infarct area is "ischemic tissue potentially destined for infarction but not yet irreversibly injured and the target of acute therapies."[16] The area of infarcted tissue in the brain is one of the main results of stroke. Ishrat et al. (2009) declared that a short course of postinjury treatment with PROG reduces cortical infarct volume and leads to an attenuation of the deficits after MCAO in rats.[17]

We also analyzed the expression levels of NMDA receptor subunits namely NR1, NR2A, NR2B, NR3A, and NR3B in the peri-infarct area after treating animals with PROG immediately after stroke. Our data revealed that the expression of NMDAR subunits NR1, NR2A, and NR2B was downregulated after tMCAO significantly, while the expression of NR3B was highly upregulated in the peri-infarct area compared to control. Also, the expression of NMDA subunits was analyzed in the presence of PROG. Our data revealed that subunits NR1, NR2A, and NR3B were significantly upregulated in the presence of PROG after stroke in peri-infarct area. Expression of NR2B did not change after PROG treatment whereas NR3A subunit was downregulated significantly.

There are a variety of different subtypes of NMDAR due to their multiple combinations of individual subunits (NR1, NR2A-D, and NR3A-B) which are coded by seven genes. Sensitivity to Mg^{2+} ion, calcium permeability, and glutamate affinity of a NMDAR, is partly determined by its subunit composition.[18] Indeed, NMDAR subunit composition and location differentially regulates neuronal survival or death.[4] Evidence suggests that synaptic NMDARs are involved in neuronal survival while the extrasynaptic NMDAR activity is correlated with cell death pathway.[19] In synapses of the adults' brain, NR2A containing NMDARs are predominant; while extrasynaptic NMDARs predominantly contain NR2B.[20] Investigations have shown that following ischemia and NDMAR activation, NR2B-containing receptors promote neuronal death signals,[20, 21] while NR2A and NR3 subunit containing receptors mediate neuronal survival signaling pathways.[19, 22] In ischemic brain, Ca^{2+}-dependent protease calpain, cleaves NR2A and NR2B subunits at their C-terminal domains[23] and serves as a negative feedback molecule to NMDA function.[24] The NR3A and NR3B subunits act as dominant negative regulators of the NMDAR current and have been shown to reduce the Ca^{2+} permeability of the NMDAR and alter Mg^{2+} sensitivity. The NR3A subunit has been shown to form excitatory glycinergic receptor complexes with NR1 alone and glutamatergic receptor complexes with NR1 and NR2 subunits. Exogenously added NR3A increases neuroprotection and endogenous NR3A protects neurons.[22] The distribution of NR3B appears to be as ubiquitous as NR1 and it is found to be expressed in motor neurons of the spinal cord, different cell types of the cerebellum, interneurons of the striatum, projection neurons, and layers of the cerebral cortex and all substructures of the hippocampus.[25,26] NR3B may modulate the function of NMDARs in somatic motor neurons during adulthood by controlling membrane trafficking and by reducing Ca^{2+} permeability. Since the high Ca^{2+} permeability is a key feature for NMDARs to play critical roles in neurodevelopment, synaptic plasticity, and neuronal death, NR3B may contribute to the regulation of these physiological and pathological processes.[27] NR3B, when exogenously introduced into hippocampal neurons, can co-assemble with endogenous NR1 and NR2A and can reduce the Ca2+ permeability of NMDA currents. In contrast, NR3B is not involved in the excitatory glycine

response in neurons. The major regulatory subunit of NMDARs is likely to switch from NR2 to NR3B in somatic motoneurons during the early postnatal period.[28] PROG provides multiple mechanisms of neuroprotection including alteration of gene expression profile after stroke in peri-infarct area.[6] This prompted us to evaluate the expression levels of NMDAR subunits by means of RT-PCR. Our results showed that PROG elevates NR1, NR2A, and NR3B subunit expression, among them NR2A and NR3B are neuroprotective and NR1 is crucial for the assembly of NMDARs in all areas of the brain. Although NR3A is a protective factor, PROG reduced its expression after ischemia. PROG did not upregulate NR2B subunit expression which promotes neuronal death. Then, PROG may do some parts of its neuroprotective function by elevation of NMDAR subunits that are involved in neuronal survival.

A variety of biochemical and molecular alterations occur in the place following the stroke. Excessive intracellular Ca^{2+} increases through glutamate receptor activation, particularly NMDAR, which can cause mitochondrial ultrastructural changes and a calcium-dependent opening of mitochondrial permeability transition pore.[29] The Ca^{2+} influx through the opened NMDAR into the postsynaptic cell activates second messengers which are responsible for long-lasting synaptic plasticity.[30] Excitotoxicity and Ca^{2+} overload are major factors contributing to the early stages of ischemic cell death. If glutamate neurotransmitter accumulates into the extracellular space, it will lead to prolonged stimulation of NMDAR subtypes to dramatically enhance the influx of calcium into neurons that it activates catabolic processes mediated by proteases, lipases, and nucleases. In the case of steroids and NMDARs, Boon et al.'s (2005) findings showed that selective loss of estrogen synthesis is associated with changes in NMDAR subunit expression in the hippocampus.[31] Also, it is shown that estradiol and selective estrogen receptor modulators could modulate NR1 and NR2B subunits in ovariectomized rats.[32] The steroid hormone PROG also has effects on NMDAR and inhibits receptor-gated rise in intracellular Ca^{2+} after brain injury that is expected to result in the prevention of neuronal death. PROG has been shown to attenuate the rise of intracellular calcium by its effects on both the receptor-gated and the voltage-gated channels after focal cerebral ischemia in vivo or in striatal neurons in vitro.[33] Indeed, PROG provides multiple mechanisms of neuroprotection that could be very important after stroke, such as reducing cerebral edema, as well as anti-inflammatory, antioxidant, antiapoptotic, and antiexcitatory properties.[6]

The heteromeric NR1/NR2 receptors activation requires the concurrent binding of two glycine molecules and two glutamate molecules.[34] These activated molecules are permeable for K^+ and Na^+ ions and also have a great efficiency for Ca^{2+}. It is interesting that NR1, NR3A, and/or NR3B subunits can create the functional purely glycine-activated channels that furthermore vary from NR1/NR2-containing receptors in other features, including low calcium permeability.[5] Therefore, increasing the expression of NR3B in improving the stroke after PROG therapy seems reasonable. Neurosteroids regulate gene expression through specific DNA-binding sites. Two hormone response elements are well known for the steroid receptors: AGGTCA is desired for estrogen receptor, and AGAACA is desired for the glucocorticoid receptors, which includes mineralocorticoid receptor, androgen receptor, and PROG receptor.[35, 36] The up-stream region of *NR3B* gene contains the defined hormone response elements (AGAACA) and this could confirm the upregulation of *NR3B* by PROG.

This study was designed to evaluate neuroprotective effects of steroid in the acute phase of the stroke; therefore, we analyzed infarction size and behavioral test 24 hours after tMCAO. However, steroid hormones such as estrogen and PROG could have long-term neuroprotective effects. Ulbrich et al. (2012) reported that estrogen and PROG could have protective effects on infarction volume and behavioral parameters 7 days and 14 days after tMCAO. Their outcomes propose that the neuroprotective effectiveness of estrogen and PROG is continued and perseveres for at least 14 days. Also, antiapoptotic and anti-inflammatory actions and angiogenesis in the injured region seems to be primarily affected early after tMCAO and is demonstrated up to 14 days.[37]

Assessing the molecular interactions through experiential tests is a very difficult procedure.[38] But the use of computational devices can be a worthy method to overcome this issue.[39] Therefore, in another part of our study, we performed a molecular docking approach to analyze the interaction of PROG with different subunits of NMDAR. Briefly, our data revealed that PROG could interact with NR1/NR2B and NR2A subunits, strongly. NMDARs are modulated by a variety of synthetic and endogenous steroids displaying potentiating or inhibitory effects.[40-42] The general trait of all steroid compounds that modulate the function of NMDAR is the existence of a charged group on the carbon C3 belonging to the steroid core.[43] Therefore, the interactions of PROG with NMDAR may modulate the effects of NMDAR during tMCAO. So estrogen can play a dual role here: (1) alteration of NMDAR gene expression and (2) modulation of NMDAR function.

Despite the extent evidence for the beneficial effects of PROG following insults such as traumatic brain injury, stroke, and excitotoxicity, the exact mechanism (s) by which PROG exerts these neuroprotective effects remain elusive.[44] These findings suggest that the acute neuroprotection of PROG is involved in NMDAR-Ca^{2+} influx.

We should mention that the absence of the analyses in a protein level of NMDAR subunits is the main limitation of this study. In conclusion, the PROG-treated ischemic rats showed significantly improved behavioral outcomes and reduced infarct volume. After MCAO the expression of NMDAR subunits NR1, NR2A, and NR2B were down-regulated, while the expression of NR3B upregulated significantly. The treatment of PROG resulted in

upregulation of NR1, NR2A, and NR3B. The NR3A expression was not influenced significantly. Our study showed that increasing the expression of downregulated NMDAR subunits may be part of a possible neuroprotective mechanism of PROG in ischemic rats.

Conflict of Interest: The authors declare that there are no conflicts of interest.

References

1. McDonald JW, Johnston MV. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res Rev 1990;15:41-70.
2. White BC, Sullivan JM, DeGracia DJ, et al. Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. J Neurol Sci 2000;179:1-33.
3. Moskowitz MA, Lo EH, Iadecola C. The science of stroke: mechanisms in search of treatments. Neuron 2010;67:181-198.
4. Stanika RI, Pivovarova NB, Brantner CA, et al. Coupling diverse routes of calcium entry to mitochondrial dysfunction and glutamate excitotoxicity. Proc Natl Acad Sci USA 2009;106:9854-9859.
5. Korinek M, Kapras V, Vyklicky V, et al. Neurosteroid modulation of N-methyl-D-aspartate receptors: molecular mechanism and behavioral effects. Steroids 2011;76:1409-1418.
6. Robertson CL, Fidan E, Stanley RM. Progesterone for neuroprotection in pediatric traumatic brain injury. Pediatr. Crit Care Med 2015;16:236.
7. Vahidinia Z, Alipour N, Atlasi MA, et al. Gonadal steroids block the calpain-1-dependent intrinsic pathway of apoptosis in an experimental rat stroke model. Neurol Res 2017;39:54-64.
8. Garcia JH, Wagner S, Liu KF, et al. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Stroke 1995;26:627-635.
9. Kramer M, Dang J, Baertling F, et al. TTC staining of damaged brain areas after MCA occlusion in the rat does not constrict quantitative gene and protein analyses. J Neurosci Methods 2010;187:84-89.
10. Lammerding L, Slowik A, Johann S, Beyer C, Zendedel A. Poststroke inflammasome expression and regulation in the peri-infarct area by gonadal steroids after transient focal ischemia in the rat brain. Neuroendocrinology 2016;103:460-475.
11. Dang J, Mitkari B, Kipp M, et al. Gonadal steroids prevent cell damage and stimulate behavioral recovery after transient middle cerebral artery occlusion in male and female rats. Brain Behav Immun 2011;25:715-726.
12. Azami-Tameh A, Clarner T, Beyer C, et al. Regional regulation of glutamate signaling during cuprizone-induced demyelination in the brain. Ann Anat 2013;195:415-423.
13. Go AS, Mozaffarian D, Roger VL, et al. Heart disease and stroke statistics—2014 update: a report from the American Heart Association. Circulation 2014;129:e28.
14. Chen J, Chopp M, Li Y. Neuroprotective effects of progesterone after transient middle cerebral artery occlusion in rat. J Neurol Sci 1999;171:24-30.
15. Kumon Y, Kim SC, Tompkins P, et al. Neuroprotective effect of postischemic administration of progesterone in spontaneously hypertensive rats with focal cerebral ischemia. J Neurosurg 2000;92:848-952.
16. Fisher M, Bastan B. Identifying and utilizing the ischemic penumbra. Neurology 2012;79:S79-S85.
17. Ishrat T, Sayeed I, Atif F, et al. Effects of progesterone administration on infarct volume and functional deficits following permanent focal cerebral ischemia in rats. Brain Res 2009;1257:94-101.
18. Zito K, Scheuss V. NMDA receptor function and physiological modulation. Encyclopedia Neuroscience 2009;6:1157-1164.
19. Liu Y, Wong TP, Aarts M, et al. NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. J Neurosci 2007;27:2846-2857.
20. Tu W, Xu X, Peng L, et al. DAPK1 interaction with NMDA receptor NR2B subunits mediates brain damage in stroke. Cell 2010;140:222-234.
21. Chen M, Lu TJ, Chen XJ, et al. Differential roles of NMDA receptor subtypes in ischemic neuronal cell death and ischemic tolerance. Stroke 2008;39:3042-3048.
22. Nakanishi N, Tu S, Shin Y, et al. Neuroprotection by the NR3A subunit of the NMDA receptor. J Neurosci 2009;29:5260-5265.
23. Bi R, Bi X, Baudry M. Phosphorylation regulates calpain-mediated truncation of glutamate ionotropic receptors. Brain Res 1998;797:154-158.
24. Guttmann RP, Sokol S, Baker DL, et al. Proteolysis of the N-methyl-D-aspartate receptor by calpain in situ. J. Pharmacol. Exp Ther 2002;302:1023-1030.
25. Al-Hallaq RA, Jarabek BR, Fu Z, et al. Association of NR3A with the N-methyl-D-aspartate receptor NR1 and NR2 Subunits. Mol Pharmacol 2002;62:1119-1127.
26. Wee KSL, Zhang Y, Khanna S, et al. Immunolocalization of NMDA receptor subunit NR3B in selected structures in the rat forebrain, cerebellum, and lumbar spinal cord. J Comp Neurol 2008;509:118-135.
27. Matsuda K, Kamiya Y, Matsuda S, et al. Cloning and characterization of a novel NMDA receptor subunit NR3B: a dominant subunit that reduces calcium permeability. Mol Brain Res 2002;100:43-52.
28. Fukaya M, Hayashi Y, Watanabe M. NR2 to NR3B subunit switchover of NMDA receptors in early postnatal motoneurons. Eur J Neurosci 2005;21:1432-1436.
29. Szydlowska K, Tymianski M. Calcium, ischemia and excitotoxicity. Cell Calcium 2010;47:122-129.
30. Dingledine R, Borges K, Bowie D. et al. The glutamate receptor ion channels. Pharmacol Rev 1999;51:7-62.
31. Boon WC, Diepstraten J, van der Burg J, et al. Hippocampal NMDA receptor subunit expression and watermaze learning in estrogen deficient female mice. Mol Brain Res 2005;140:127-132.
32. Cyr M, Ghribi O, Thibault C, et al. Ovarian steroids and selective estrogen receptor modulators activity on rat brain NMDA and AMPA receptors. Brain Res Rev 2001;37:153-161.
33. Cai W, Zhu Y, Furuya K, et al. Two different molecular mechanisms underlying progesterone neuroprotection against ischemic brain damage. Neuropharmacology 2008;55:127-138.
34. Furukawa H, Singh SK, Mancusso R, Gouaux E. Subunit arrangement and function in NMDA receptors. Nature 2005;438:185-192.
35. Mangelsdorf DJ, Thummel C, Beato M, et al. The nuclear receptor superfamily: the second decade. Cell 1995;83:835-839.
36. Roemer SC, Donham DC, Sherman L, et al. Structure of the progesterone receptor-deoxyribonucleic acid complex:

novel interactions required for binding to half-site response elements. Mol Endocrinol 2006;20:3042-3052.
37. Ulbrich C, Zendedel A, Habib P, et al. Long-term cerebral cortex protection and behavioral stabilization by gonadal steroid hormones after transient focal hypoxia. J Steroid Biochem Mol Biol 2012;131:10-16.
38. Soleimani Z, Kheirkhah D, Sharif MR, et al. Association of CCND1 gene c.870 G>a polymorphism with breast cancer risk: a case-control study and a meta-analysis. Pathol Oncol Res 2017;23:621-631.
39. Mazaheri M, Karimian M, Behjati M, et al. Association analysis of rs1049255 and rs4673 transitions in p22phox gene with coronary artery disease: A case-control study and a computational analysis. Ir J Med Sci 2017;186:921-928.
40. Park-Chung M, Wu FS, Farb DH. 3 alpha-Hydroxy-5 beta-pregnan-20-one sulfate: a negative modulator of the NMDA-induced current in cultured neurons. Mol Pharmacol 1994;46:146-150.
41. Weaver CE, Land MB, Purdy RH, et al. Geometry and charge determine pharmacological effects of steroids on N-methyl-D-aspartate receptor-induced Ca^{2+} accumulation and cell death. J Pharmacol Exp Ther 2000;293:747-754.
42. Bowlby MR. Pregnenolone sulfate potentiation of N-methyl-D-aspartate receptor channels in hippocampal neurons. Mol Pharmacol 1993;43:813-819.
43. Park-Chung M, Wu FS, Purdy RH, et al. Distinct sites for inverse modulation of N-methyl-D-aspartate receptors by sulfated steroids. Mol Pharmacol 1997;52:1113-1123.
44. Luoma JI, Stern CM, Mermelstein PG. Progesterone inhibition of neuronal calcium signaling underlies aspects of progesterone-mediated neuroprotection. J Steroid Biochem Mol Biol 2012;131:30-36.